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Review

Sphingosine-1-phosphate transport and its role in immunology

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Abstract: Sphingosine-1-phosphate (S1P) is a sphingolipid metabolite with many important functions in cellular and systemic physiology, including the immune system. As it cannot traverse the membrane, it is exported from cells by transporters. Several members of the ATP-binding cassette (ABC) transporter family, ABCA1, ABCC1, ABCG2 and potentially ABCA7 have been identified as S1P transporters. In addition spinster 2 (SPNS2), a protein from the major facilitator superfamily (MFS), was identified as a S1P transporter. Here we review the current knowledge on S1P transport and discuss how this process creates S1P gradients in the body that are important in various functions of the immune system.

Keywords: ABC transporter; SPNS2, S1P; immunology; leukocyte

Abbreviations:

ABC = ATP-binding cassette; CDase = ceramidase; DC = dendritic cell; HEV = high endothelial venule; MFS = major facilitator superfamily; NET = neutrophil extracellular trap; PRR = pattern-recognition receptor; SMase = sphingomyelinase; S1P = sphingosine-1-phosphate; S1PR = sphingosine-1-phosphate receptor; SphK = sphingosine kinase; SPL = sphingosine-1-phosphate lyase; SPNS2 = spinster 2; SPP = sphingosine-1-phosphate phosphohydrolase; SR-B1 = scavenger receptor class B1

1. Transport of S1P

Local production and export of sphingosine-1-phosphate (S1P) can generate concentration gradients of this bioactive sphingolipid in the body [1,2]. Given the potential role for S1P transporters in establishing such gradients, it is important to identify the molecular nature of these transporters. Both ATP-binding cassette (ABC) transporters and spinster 2 (SPNS2) have been revealed as transporters able to move S1P over the cell membrane.

1.1. ABC transporter-mediated transport of S1P

The family of ABC transporters is an important group of membrane-associated proteins that serve as transmembrane transporters for various substrates, such as lipids, cytokines and cytostatics. Of the 48 human ABC transporters almost half are thought to translocate lipids or lipid-related compounds, including phospholipids, cholesterol and sphingolipids [3]. S1P is an important sphingolipid metabolite with well-established signaling functions. It is generated in the sphingolipid catabolic pathway by phosphorylation of sphingosine through the action of either of two sphingosine kinases (SphK), SphK1 and SphK2, of which SphK1 acts at the plasma membrane (Figure 1A) [4]. Sphingomyelin (SM) is a source for generation of S1P in the plasma membrane and is initially converted to sphingosine by the subsequent actions of the enzymes sphingomyelinase (SMase), which generates ceramide, and ceramidase (CDase; Figure 1A). SM is considered to localize in the exoplasmic leaflet of the plasma membrane and is not prone to flipping to the cytoplasmic leaflet. Both SMase and CDase act on the exoplasmic leaflet and together generate sphingosine [5,6]. Sphingosine therefore has to flip to the cytoplasmic leaflet of the plasma membrane to allow SphK1 to convert it into S1P. Catabolism of S1P inside the cell by S1P lyase (SPL) leads to the formation of hexadecenal and phosphoethanolamine [7]. Alternatively, S1P is dephosphorylated to sphingosine, for which several phosphatases are known to exist [8]. So, at least 5 enzymes together determine the (intra)cellular levels of S1P (Figure 1A).

Apart from intracellular biological significance of S1P, the lipid exerts important functions by interacting with S1P receptors on the cell surface, of which 5 types exist, S1PR₁₋₅ [9]. Through these receptors S1P can generate a plethora of signals via autocrine and/or paracrine signaling leading to various biological outcomes, such as cell survival, proliferation, differentiation, migration and immune responses. For example, local production and export of S1P can generate a concentration gradient between different compartments that can be chemotactic to subtypes of leukocytes, thereby stimulating their migration against the gradient of S1P (see also sections 2 and 3). Systemic transport of S1P with the blood flow occurs in complexed form bound to HDL or albumin (Figure 1A) [10,11].

In order to exert its extracellular functions, intracellularly generated S1P needs to be transported over the membrane by an active or passive transporter, since the molecule is too hydrophilic to simply diffuse over the membrane (Figure 1A). This is where ABC transporters come into play with the original discovery of ABCC1 as transporter for S1P in human and rodent mast cells [12] (Table S1). Release of S1P through ABCC1 was demonstrated pharmacologically, using the ABCC1 inhibitor MK571, and using a molecular approach involving downregulation of ABCC1 [12]. It is thought that S1P release regulates migration of mast cells towards antigen (see also section 3.3). S1P is released

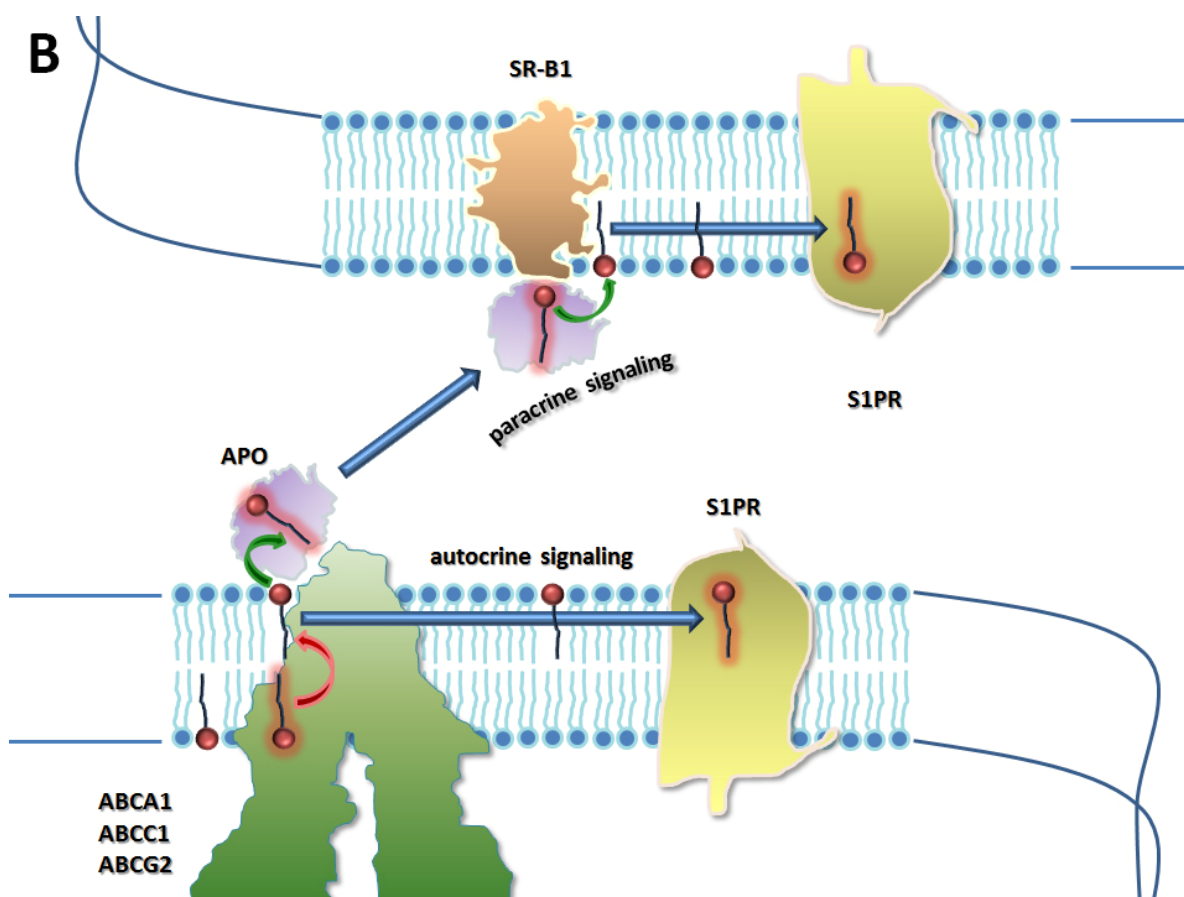
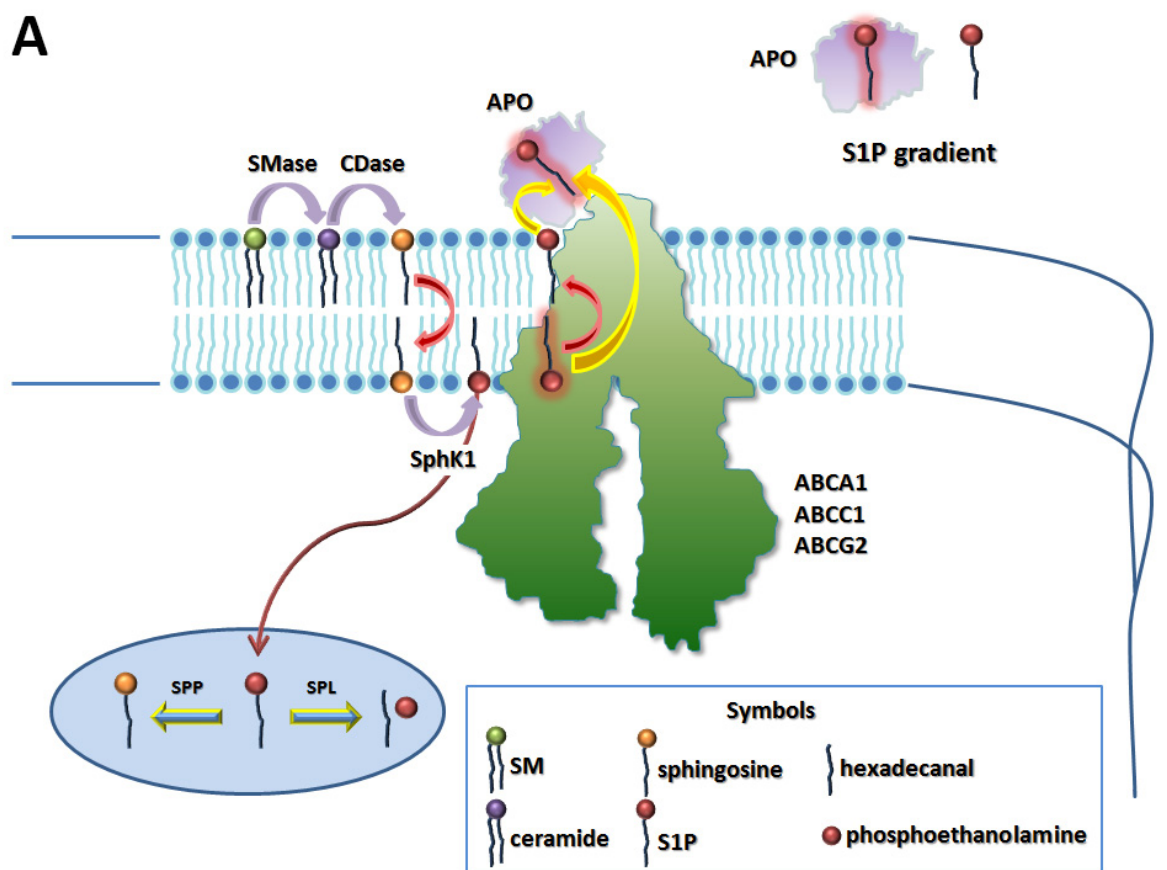


Figure 1. S1P-centered metabolism, transport and its topology. A) In the plasma membrane sphingomyelin (SM) is sequentially converted to ceramide, sphingosine and sphingosine-1-phosphate (S1P) by sphingomyelinase (SMase), ceramidase (CDase) and sphingosine kinase (SphK1), respectively. SM is considered to localize in the exoplasmic leaflet and is not prone to flipping. SMase and CDase act on the exoplasmic leaflet, so sphingosine has to flip to the cytoplasmic leaflet of the plasma membrane, where SphK1 can act on it to produce S1P. S1P flops to the exoplasmic leaflet due to the action of ABC transporters, which have a lateral gate for lipids/lipophilic molecules in the plane of the membrane. Subsequently, S1P may be loaded onto APO proteins in the process of HDL particle formation. Alternatively, S1P is picked up from the cytoplasmic leaflet and directly loaded on the extracellular acceptor (HDL) by the ABC transporter. S1P outside the cell will be bound to HDL (or albumin) in equilibrium with a free pool of S1P and as such may generate a gradient from the blood circulation towards tissues with low S1P levels. Alternatively, S1P can be dephosphorylated to sphingosine by S1P phosphatases (SPP) or catabolized to hexadecanal and phosphoethanolamine by S1P lyase (SPL). B) After flopping to the exoplasmic leaflet, S1P can diffuse laterally and engage in binding to the lipid binding pocket of a S1P receptor (S1PR), which is located in the plane of the membrane, thus activating S1PR (autocrine signaling). Alternatively, extracellular S1P, be it soluble or HDL-bound, can travel to another cell, followed by incorporation into the exoplasmic plasma membrane of that cell, e.g. after binding of HDL to its receptor, SR-B1. There, S1P can similarly activate S1PR from within the membrane (paracrine signaling).

constitutively from mast cells, but is enhanced by antigen stimulation, while both the constitutive and the stimulated release depend on ABCC1. In addition, antigen stimulation activates SphK and stimulates its translocation to the plasma membrane, thereby generating efficient production/export machinery together with ABCC1 at the plasma membrane (Figure 1A) [12]. The function of ABCC1 as a S1P transporter was confirmed in several studies. ABCC1 was shown to export S1P in rat uterine leiomyoma ELT3 cells and late pregnant rat myometrium, since release is inhibited by MK571 or after downregulation by ABCC1 siRNA [13]. It was concluded that ABCC1 is involved in 4β -phorbol 12,13-dibutyrate-induced COX2 expression mediated by SphK1/S1P which may contribute to leiomyoma growth. ABCC1 was also implicated in dexamethasone-mediated protection of human fibroblasts against (TNF-mediated) programmed cell death [14]. Both MK571 and ABCC1 siRNA abrogate the cytoprotective effect of dexamethasone, while ABCC1 siRNA treatment results in increased cellular S1P levels. Moreover, ABCC1 expression is upregulated by dexamethasone [14]. In murine blood-brain and blood-spinal cord barriers ABCC1 was identified as an important link between TNF α signaling and S1PR₁-dependent reduction of ABCB1 activity [15]. Capillaries isolated from homozygous ABCC1-deficient mice do respond to S1P but not to TNF α in terms of reduced ABCB1 activity, while MK571 inhibits sphingosine-mediated reduction of ABCB1 activity. This study places ABCC1 central in inside-out signaling via S1P export leading to S1PR₁ activation and subsequent effects on ABCB1 [15].

Apart from ABCC1, the ABC transporters ABCA1 and ABCG2 have also been implicated in S1P transport. In MCF7 human breast cancer cells estradiol stimulates S1P export from cells and this effect is suppressed by pharmacological inhibition or gene silencing of *ABCC1* or *ABCG2* [16] (Table S1). The secreted S1P may be involved in cancer progression and/or multidrug resistance of cancer cells. Retinoic acid/cAMP treatment of astrocytes induces extracellular S1P accumulation in HDL particles, which is inhibited by either ABCA1 siRNA treatment or the non-selective ABCA1 inhibitor glibenclamide [17] (Table S1). Moreover, astrocytes from *Abca1* homozygous null mice display low S1P release in response to retinoic acid/cAMP treatment [17]. S1P release from human vascular endothelial cells is inhibited by MK571 as well as glibenclamide, suggesting involvement of ABCC1 and ABCA1 [18]. However, plasma levels of S1P from *Abca1*, *Abca7* or *Abcc1* homozygous null mice do not show significant changes compared to wild-type mice [18]. This finding may indicate compensation by other transporters, for example SPNS2 (see also section 1.2). In this context, expression of functional ABCA1, ABCB1, ABCC1 or ABCG2 transporters in CHO/SphK1 cells does not lead to secretion of S1P in the medium of these cells, suggesting that ABC transporters alone are not sufficient to function as S1P transporters [19]. ABC transporters may need to interact with other proteins that could be absent in the CHO/SphK1 expression system. Alternatively, the lack of effect may be due to cell type specific modifications of ABC transporters in CHO cells.

Given the potential role for blood and endothelial cells in establishing S1P gradients in the body, it is relevant to discuss the S1P transporters involved in these processes. Plasma and lymph have distinct sources that supply S1P and are maintained as separate compartments in this respect. High levels of S1P in plasma are generated by export of S1P from erythrocytes, platelets and endothelial cells, although the contribution of platelets appears to be small [11,20-25]. Erythrocytes are considered to be the major producer of plasma S1P (see also section 2). Lymphatic endothelial cells are likely the source of S1P in lymph [26]. Platelets appear to have two independent S1P transporters. One is ATP-dependent, stimulated by thrombin and inhibited by glibenclamide, but not MK571. This transporter was suggested to be ABCA1 and/or ABCA7 [27] (Table S1). The other transporter is stimulated by Ca^{2+} and not inhibited by glibenclamide [27]. In contrast to platelets, erythrocytes constitutively release S1P and do not depend on stimuli to release S1P. S1P release by erythrocytes is inhibited by glibenclamide [28]. Moreover, transport of S1P into rat erythrocyte inside-out membrane vesicles is also selectively inhibited by glibenclamide [28]. The effect of MK571 on blockade of S1P transport from erythrocytes, however, is conflicting. Previously, partial inhibition of S1P transport from erythrocytes by glibenclamide and MK571, either alone or combined, was demonstrated [29]. Taken together, the results suggest that S1P export from erythrocytes involves an ABCA subfamily member; ABCA7 is a more likely candidate than ABCA1, as the latter is not detectable in the erythrocyte vesicle preparation [28]. Involvement of ABCC1 needs to be further investigated. Interestingly, the uptake of S1P into erythrocyte inside-out membrane vesicles is supported not only by ATP, but also by dATP and AMP-PNP and not by ATP γ S. This indicates a dependence of the transporter on adenine nucleotides with an intact γ -phosphate group, but no requirement for ATP hydrolysis [28].

Most of the studies on the involvement of ABC transporters in S1P export from cells rely on the use of pharmacological inhibitors, such as glibenclamide, which are not 100% specific [30] and thus may have off-target effects. Fortunately, in most cases the findings with pharmacological inhibitors are

validated with siRNA-knockdown in cell lines or gene targeting in mice. It should be noted that an alternative mechanism to generate extracellular S1P exists, that does not rely on export of intracellularly generated S1P. In this case the enzyme SphK1 is exported from vascular endothelial cells, is enzymatically active outside the cell and generates physiologically relevant amounts of S1P for S1PR stimulation. This mechanism may contribute to the establishment of the vascular S1P gradient [31,32].

Several models have been proposed for the mechanism of function of ABC transporters. Although ABC transporters were originally perceived as pore-forming proteins with an aqueous pore that acts as a channel allowing the movement of hydrophilic substrates across the membrane, it is now considered to function as a floppase, moving lipid soluble molecules from the inner to the outer plasma membrane leaflet. Combining this function with a potential loading function for substrates onto acceptors, provides the rationale for several models by which ABC proteins are perfectly fit for export of lipids, including S1P. As such they are now thought to be an essential link in S1P inside-out signaling. First, localization of S1P in the outer leaflet after flopping would be compatible with an autocrine function of the lipid by binding to a S1PR. Indeed, it is thought that S1P does not approach the receptor from the aqueous environment, but rather sideways in the plane of the membrane, consistent with the presence of an intramembranous lipid access channel in the receptor (Figure 1B) [33]. Second, for paracrine signaling functions S1P needs to be released into the aqueous environment. ABC transporters are considered to perform such a function by picking up the substrate (i.e. S1P) from the inner leaflet and loading it onto an acceptor molecule such as APO (in HDL) or albumin (Figure 1B). Third, S1P could be picked up from the outer leaflet and loaded on an extracellular acceptor (Figure 1B) [3].

1.2. SPNS2-mediated transport of S1P

In 2008/2009, a novel S1P transporter, SPNS2, was identified by two groups independently [34,35] (Table S1). Kawahara et al. investigated the features of the zebrafish *ko157* mutant [35]. A point mutation (R153S) in the gene encoding SPNS2 results in cardia bifida in zebrafish as a result of defective migration of myocardial precursors. This effect is likely mediated by the S1P/S1PR₂ pathway, since the *miles apart (mil)/S1PR₂* mutant with a mutation in the gene encoding S1PR₂ shows the same phenotype. Evidence is provided for an interaction between the genes coding for SPNS2 and S1PR₂. Rescue experiments reveal that SPNS2 function is conserved from fish to mammals and suggest that SPNS2 functions upstream of S1PR₂. Both zebrafish SPNS2-EGFP and human SPNS2-EGFP induce S1P export when expressed in CHO/SphK1 cells, in contrast to mutated variants [35]. FTY720 is a synthetic sphingosine analog that, after being phosphorylated, binds to and activates S1PR₁, S1PR₃, S1PR₄ and S1PR₅, but does not interact with S1PR₂ [36]. Hisano et al. investigated the substrate specificity of human SPNS2 expressed in the CHO/SphK1 system and report that apart from S1P also FTY720, dihydro-S1P, and phyto-S1P are transported. This was the first identification of a transporter for FTY720 and shows that FTY720 uses the same transporter as S1P [19]. Dihydro-S1P transport by SPNS2 was confirmed in another study [37].

S1P transport was subsequently studied by various groups in *Spns2*-deficient mice, i.e. global or targeted disruption of *Spns2* [37-41]. All studies agree that SPNS2 is important for S1P release from

endothelial cells, which in two studies is demonstrated using endothelial-specific knockouts of *Spns2* [38,40]. SPNS2 is not involved in S1P release from erythrocytes [38,39] or platelets [39]. The murine studies further demonstrate that knock out of *Spns2* results in a different phenotype from zebrafish. Zebrafish present with a prominent cardiovascular phenotype while mice lack developmental defects but show a prominent lymphocyte trafficking phenotype. This indicates divergence in expression and/or functions of SPNS2 between these species and could mean potential divergence in humans as well. SPNS2 may well be involved in many other cell types and processes. Recently it was shown that SPNS2 is expressed in murine osteoblastic MC3T3 cells and its downregulation by siRNA treatment results in impaired paracrine signaling towards prostate cancer cells, which are supported by S1P in terms of proliferation and survival [42].

The molecular structure of SPNS2 is unknown and awaits crystallization of the protein and high-resolution structure determination. Based on the predicted amino acid sequence, it belongs to the major facilitator superfamily (MFS) of transporters [10]. These V-shaped transporters have 12 transmembrane helices and recognize hydrophilic substrates. As discussed, S1P is lipophilic and likely approaches membrane proteins laterally in the plane of the membrane, as is the case for ABC transporters and S1PR₁ (Figure 1B). The shape of MFS transporters could be compatible with such a function, as it shows similarities with the V-shaped ABC transporters with 12 transmembrane helices. It can be speculated that SPNS2 similar to ABC transporters has a lateral substrate access gate in the plane of the membrane.

In conclusion, various members of the ABC transporter family on the one hand and SPNS2 on the other hand have so far been identified as S1P transporters. For ABCC1 this has been established in various cell systems. Members of the ABCA subfamily are implicated as well, but this needs further confirmation, especially regarding ABCA7. Given their expression on blood and endothelial cells and the inducible expression of S1PRs in leukocytes, S1P transporters play an important role in leukocyte trafficking, as will be discussed below.

2. Involvement of S1P in lymphocyte trafficking

Release of matured T cells and immature B cells from thymus and bone marrow, respectively, appears to be regulated by SPNS2. Mice deficient for *Spns2* in endothelial cells have a reduced egress of T cells from the thymus and B cells from the bone marrow [38]. Upon maturation, T cells upregulate the expression of S1PR₁ and migrate from the thymus into the circulation, stimulated by the gradient of S1P produced by neural crest-derived pericytes (Figure 2A) [43]. As a consequence, mice systemically deficient for *Sphk1* or lacking *Spns2* in endothelial cells not only have a reduced number of circulating lymphocytes, but also a lower number of lymphocytes in secondary lymphoid organs [26,38]. However, although circumstantial evidence might suggest a role for SPNS2 in transporting S1P from thymic pericytes, no direct data is available yet to support this hypothesis. Cross-breeding *Spns2*-floxed mice with *Wnt1*-Cre mice will lead to specific deletion of *Spns2* in thymic pericytes and may provide direct evidence for the role of SPNS2 in release of S1P from these cells. Taken together, the available data suggest that SPNS2 plays an important role in regulating the release of T and B cells from primary lymphoid organs into the circulation.

Recirculation of (mature) lymphocytes is essential for proper adaptive immune function by

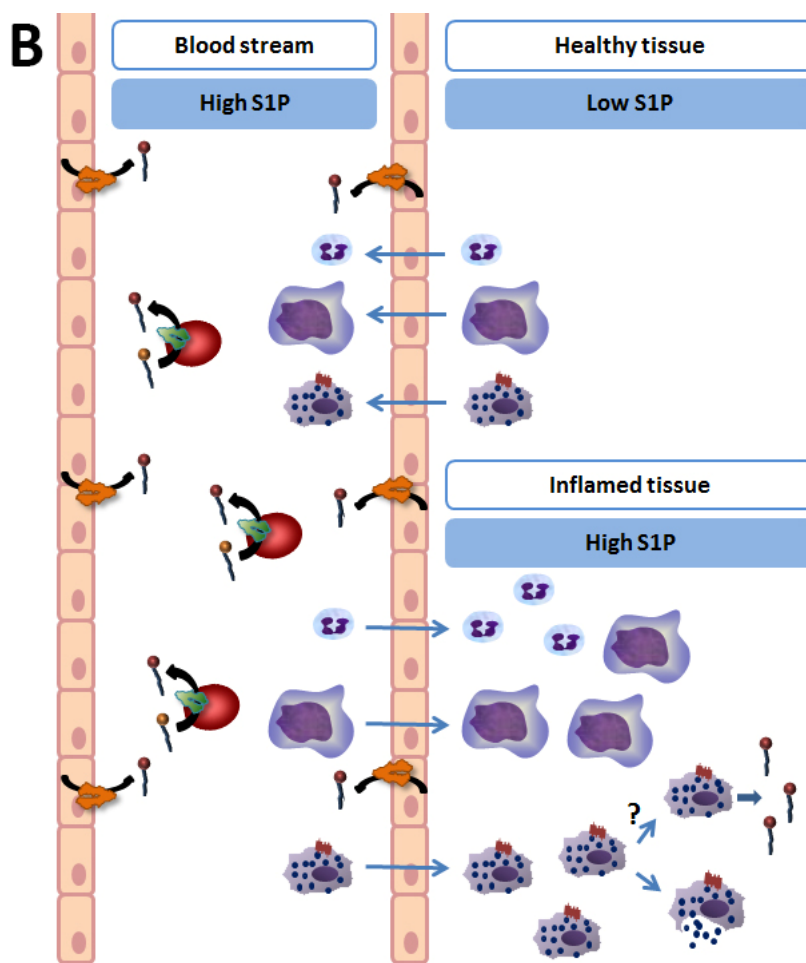
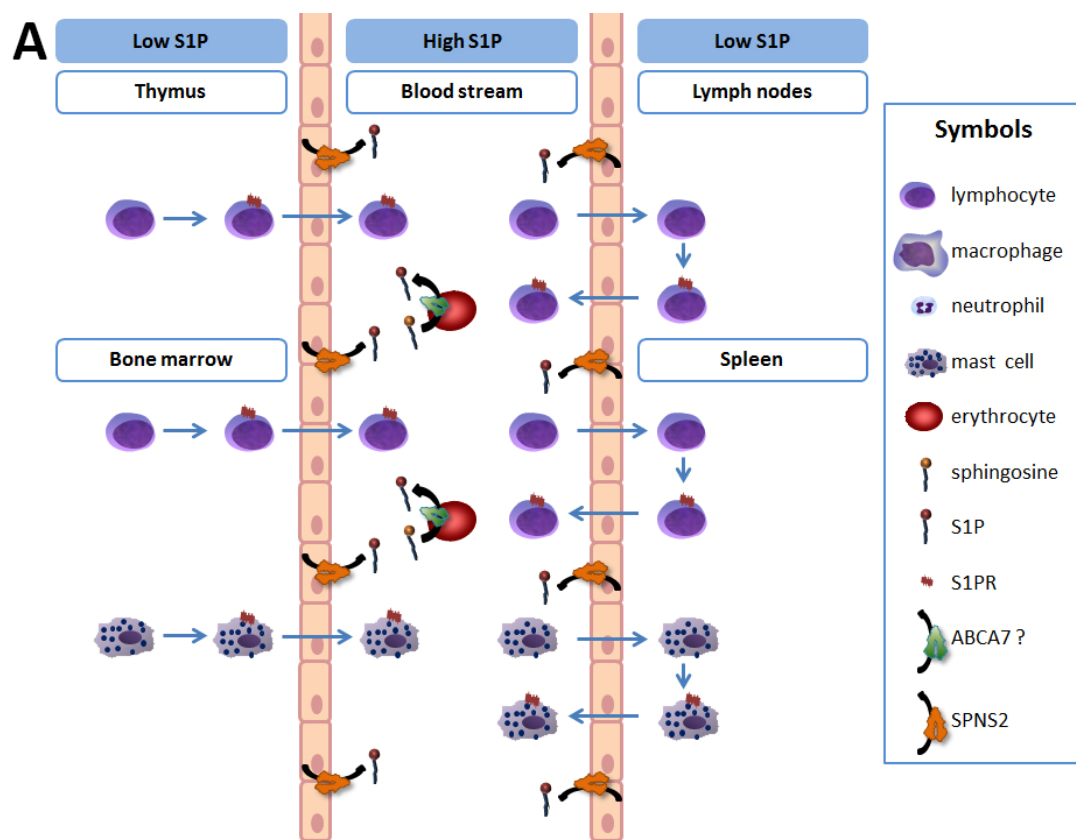


Figure 2. Effects of S1P on leukocyte migration. A) Upon maturation in primary lymphoid organs, T cells (thymus), B cells and mast cells (both in bone marrow) upregulate the expression of S1PR₁. The gradient of S1P, with relatively high levels in the plasma generated from transport of S1P from thymic pericytes (potentially by SPNS2), stimulates egress of cells into the circulation. In contrast to primary lymphoid organs, it is not clear whether it is the S1P gradient (relatively high levels of S1P in plasma) or the absolute level of S1P that stimulates egress from secondary lymphoid organs (lymph nodes and spleen). S1P is transported by SPNS2 from endothelial cells to the lymph and subsequently stimulates egress of lymphocytes and mast cells from secondary lymphoid organs through S1PR₁ signaling (not shown). Levels of S1P in plasma are mainly regulated by erythrocytes and vascular endothelial cells. Erythrocytes presumably use ABCA7 while endothelial cells use SPNS2 to transport S1P. B) An inflammatory stimulus increases the tissue S1P level (potentially produced by mast cells) and subsequently increases the migration of macrophages, neutrophils and mast cells to the inflammatory site.

allowing immune surveillance and the induction of an immune response by interaction with other leukocytes. Therefore, T and B cells recirculate by entering lymph nodes through high endothelial venules (HEVs) and leave the lymph nodes on the abluminal side into the efferent lymphatics [36]. In turn, the lymphocytes migrate back to the blood through the efferent lymphatics that eventually drain into the thoracic duct [44]. S1P is suggested to play an essential role in stimulating egress of lymphocytes from secondary lymphoid organs, as demonstrated by experimental internalization of S1PR₁ by FTY720. Injection of FTY720 leads to a significant lymphopenia after 4 hours (93% decrease in CD4⁺ and 88% decrease in CD8⁺ T cells; 90% decrease in B cells), without affecting the number of myelomonocytic cells in blood, as demonstrated in mice and rats [45,46]. FTY720 not only leads to a reduced number of lymphocytes in blood, but also in the thoracic duct [36,45], suggesting sequestration of lymphocytes within secondary lymphoid organs. Moreover, within lymph nodes FTY720 induces migration of lymphocytes from the sinuses towards the lymph node exit sites, the abluminal side of the endothelial layer [36]. Likely, loss of S1P-sensing due to internalization of the S1P receptor by FTY720 induces retention of lymphocytes in lymph nodes, lowering the number of lymphocytes in blood. In addition, it was demonstrated that a reduction in the plasma level of S1P leads to lymphopenia as well. During mammalian hibernation, S1P plasma levels are reduced due to a reduced release of S1P from erythrocytes and this leads to a 95% decrease in the number of circulating lymphocytes [29]. Studies using knockout mice suggest that S1P signaling through S1PR₁ is essential for migration of lymphocytes from secondary lymphoid organs back into the systemic circulation (Figure 2A) [1,2,26]. Thus, signaling of S1P through S1PR₁ on lymphocytes seems to play an essential role in stimulating release of lymphocytes into the circulation.

Recirculation of lymphocytes through blood and secondary lymphoid organs is suggested to primarily depend on S1P, although the exact mechanisms remain to be unraveled. As such, one hypothesis is that lymphocyte egress depends on a S1P gradient between blood and lymph, with high levels in blood and low levels in lymph (Figure 2A) [1,26]. Erythrocytes are considered to be an important source of plasma S1P since they do not possess S1P-degrading enzymes and display a high

SphK activity [26,47,48]. Transplantation of *Sphk*-deficient bone marrow from mice deficient for both *Sphk1* and *Sphk2* into wild-type mice leads to a 10-fold reduced S1P plasma level, while transplantation of *Sphk1*^{+/-}*Sphk2*^{-/-} bone marrow to irradiated wild-type mice does not affect the S1P plasma level [26]. Hence, these data show that SphK1 in erythrocytes seems to be responsible for up to 90% of S1P in plasma. In addition to a role for SphK1 in erythrocytes, experimental ablation of *Spns2* demonstrates a role for SPNS2 in endothelial cells in regulating S1P in plasma. Knockout of *Spns2* completely abolishes the S1P transport from vascular endothelial cells and leads to a 40–50% reduction in the S1P plasma level [37,39,40]. Moreover, expression of SPNS2 is barely detectable in bone marrow and *Spns2*-deficiency in mice does not affect release of S1P from erythrocytes [39]. Taken together, these results indicate that 50–90% of plasma S1P is derived from erythrocytes produced by SphK1 and is not transported by SPNS2, while 10–50% of plasma S1P is likely produced by SphK2 and released from endothelial cells by SPNS2.

Although transplantation of *Sphk*-deficient bone marrow into wild-type mice leads to a lowered S1P plasma level, the level of S1P in lymph is not affected [26]. As a consequence of the lowered S1P level in plasma, the S1P gradient between plasma and lymph is disrupted. However, the number of lymphocytes in thymus, secondary lymph nodes and blood is not different from control mice [26]. Hence, although it is hypothesized that lymphocyte egress depends on a S1P gradient between blood and lymph, current data reveal that this gradient is unlikely to be the sole factor driving lymphocyte egress [26,49,50]. Since *Sphk1*-deficient mice have a reduced S1P plasma level but no significant reduction in the number of circulating lymphocytes [26,50], local production and release of S1P in lymphoid organs seem to be the key factors in regulating lymphocyte recirculation. In addition to promoting release of lymphocytes from thymus and bone marrow (primary lymphoid organs) as described above, SPNS2 also seems to play an important role in regulating local levels of S1P in lymph and thereby stimulating lymphocyte egress from secondary lymphoid organs. Intriguingly, contradicting findings on lymph S1P levels are reported in *Spns2*-deficient mice. Mendoza et al. demonstrated that specific knockout of *Spns2* in endothelial cells leads to a reduced S1P level in plasma and lymph [40]. Nagahashi et al. on the other hand revealed that mice systemically deficient for *Spns2* have decreased levels of S1P in plasma, while the level of S1P in lymph is increased [37]. It should be noted however, that both Mendoza et al. and Nagahashi et al. used a mouse model that lacked *Spns2* in all endothelial cells, not specifically in lymphatic endothelial cells. Cross-breeding *Spns2*-floxed mice with *Lyve1*-Cre mice would lead to specific knockdown of *Spns2* in lymphatic endothelium. To our knowledge, no study thus far used such an approach to demonstrate the specific role of SPNS2 in transporting S1P from lymphatic endothelium. As described above, export of SphK may lead to the production of physiological amounts of extracellular S1P and thus may contribute to the formation of a S1P-gradient in addition to transport of S1P from cells [31,32]. Hence, in addition to transport of S1P from endothelial cells by SPNS2, extracellular SphK might well contribute to local formation of S1P in lymph.

Despite different effects on the S1P level in lymph, both systemic and endothelial-cell specific knockout of *Spns2* in mice leads to a reduced number of circulating lymphocytes. In addition, *Spns2*-deficient mice have increased numbers of T cells in thymus, especially CD4⁺ CD62L⁺ CD69[–] and CD8⁺ CD62L⁺ CD69[–], which represent matured T cells [37]. Therefore, the increased number of T cells in thymus from *Spns2* deficient mice is likely due to diminished release of these cells from the

thymus rather than secondary to overproduction of T cells, which is in line with previous studies demonstrating the essential role for SPNS2 in stimulating egress of lymphocytes from thymus and bone marrow [38]. Hence, it seems likely that lymphopenia in *Spns2*-deficient mice is secondary to inhibition of thymic egress. However, experimental ablation of the S1P-gradient between thymus and plasma by knockout of *Lipid phosphate phosphatase 3* in thymic epithelial cells or *Sphk* in neural crest-derived thymic pericytes in mice, leads to similar levels of T cell accumulation in the thymus, but only a slight reduction in the number of T cells in blood as compared to *Spns2*-deficient mice [43,51]. In contrast, knockout of *Sphk* in lymphatic endothelial cells does not affect thymic lymphocyte egress, but leads to a similar reduction in the number of circulating lymphocytes as compared to mice deficient for *Spns2* [43,52]. Therefore, the reduced number of lymphocytes in blood in *Spns2*^{-/-} mice is likely due to a combination of reduced egress from primary and secondary lymphoid organs, i.e. thymus and lymph nodes.

Taken together, it is clear that SPNS2 plays an essential role in stimulating egress of lymphocytes from primary lymphoid organs and regulates S1P in lymph by transporting S1P from endothelial cells. Although the precise effects of S1P on lymphocytes remain to be unraveled, mounting evidence suggests a major role for S1P signaling through S1PR₁ in regulating lymphocyte egress from secondary lymphoid organs.

3. Role of S1P in regulating migration and function of other leukocytes

Besides the effects of S1P signaling on lymphocytes there are effects on innate immune cell trafficking. The innate immune system usually responds quickly to an inflammatory stimulus with the infiltration of phagocytes such as neutrophils, macrophages and dendritic cells [53]. Adhesion molecules on the endothelial surface are upregulated in response to a pathogen, either directly via binding of the pathogen to an endothelial pattern-recognition receptor (PRR) or indirectly via tissue resident leukocytes that release inflammatory mediators (such as histamine and cytokines) [54]. The leukocyte-endothelial interaction leads to leukocyte influx at the inflammatory site and this involves a number of processes, including rolling, adhesion, and transmigration.

Although S1P has been shown to influence a range of cells and functions from the innate immune system most studies used pharmacological compounds, while the use of siRNA-knockdown in cell lines or gene targeting in mice has been limited. Additionally, research has focused on the role of receptors and kinases, while the investigation of the role of S1P transporters has been mostly neglected. Here, we summarize the current knowledge about S1P involvement in leukocytes other than lymphocytes.

3.1. Neutrophils

Neutrophils are among the first leukocytes to be recruited at the inflammatory site where they eliminate pathogens by phagocytosis, degranulation or neutrophil extracellular traps (NETs) [54]. An inflammatory challenge such as the injection of carrageenan (a polysaccharide) increases S1P locally and it declines when inflammation resolves [55]. It is suggested the S1P is transported from the blood into the inflamed tissue. Alternatively, mast cells that are also recruited during an inflammatory

response might contribute to the local S1P increase as hypothesized by Olivera et al. [56] since mast cells can release S1P via ABCC1 (see also sections 1.1 and 3.3; Figure 2B) [12]. Injection of carrageenan leads to neutrophil infiltration and local edema in rats, which can be completely precluded by neutralization of S1P by LT1002 (a monoclonal antibody against S1P) as well as blockage of the S1PR₁ by W123 (a S1PR₁ antagonist) [57]. S1P increases adhesion of human neutrophils to endothelium coated with suboptimal immune complex density *in vitro* [58], while injection of FTY720 reduces neutrophil rolling [59]. However, S1PR modulation by either FTY720 (effects on S1PR₁, S1PR₃, S1PR₄ and S1PR₅) or BAF312 (effects on S1PR₁ and S1PR₅) does not affect the number of neutrophils in blood [60]. Thus, neutrophil circulation seems to be independent of S1P receptors, while neutrophil infiltration depends on S1P binding via S1PR₁. Likely, reduced influx of neutrophils into tissues is secondary to inhibition of rolling and adhesion of neutrophils to the vascular endothelium.

Histamine-induced neutrophil rolling can be reduced by inhibiting sphingosine kinases and is also reduced in *Sphk1*^{-/-} mice, but not in *Sphk2*^{-/-} mice [59]. In contrast to these findings, Zemann et al. reported no differences in neutrophil circulation or function in a study with knockout mice for *Sphk1* or *Sphk2* [61]. They observed normal calcium-influx as well as normal *in vitro* migration after different stimuli. Moreover, recruitment of neutrophils into the peritoneum (following injection of the neutrophil chemokines KC and MIP-2) and peripheral blood (following injection of the chemotactic peptide fMLP) *in vivo* were normal. Thus although knockout of *Sphk1* reduces neutrophil rolling, eventual neutrophil recruitment seems independent from sphingosine kinases. Further, the number and function of neutrophils in blood does not appear to be mediated by the absolute S1P level in plasma, which is different in the two different knockout models. While *Sphk1*^{-/-} mice have a reduced plasma level S1P due to lower S1P production (see also section 2), data on the S1P plasma level in *Sphk2*^{-/-} mice are conflicting. Linke et al. show that the measured S1P plasma levels depend on centrifugation speed, which suggests release from damaged cells upon centrifugation [62]. When taking the centrifugation speed into account, the S1P plasma levels in *Sphk1*^{-/-} or *Sphk2*^{-/-} mice were not different from the S1P plasma level in wild-type mice, although the intracellular S1P level is increased in *Sphk2*^{-/-} mice. Hence, the reported increased level of S1P in plasma from *Sphk2*^{-/-} mice seems to represent a technical artefact, due to release from damaged erythrocytes. The higher level of S1P in erythrocytes might be due to a compensatory elevation of SphK1 [56,63].

The apparent contradictions between the study of Zemann et al. and other studies might be explained by a difference in tissue S1P concentration, since S1P concentrations were influenced locally in tissue in the three studies that document alterations in neutrophil function [57,58,59]. In healthy tissue S1P gradients are short-lived due to degradation of S1P by S1P lyase [64]. However, inhibition of S1P breakdown or increased release of S1P can stabilize the gradient and influence the local inflammatory response. Taken together, is it likely not the absolute S1P levels, but rather the relative S1P gradient between different compartments, such as blood and tissue, which stimulates migration and activation of neutrophils (Figure 2B). In line with this notion, Allende et al. revealed that *S1p lyase* deficient (*Sgpl1*^{-/-}) mice with permanently elevated levels of circulating S1P and increased S1P levels in tissues have neutrophilia as well as impairment of neutrophil function, including inability to migrate into tissues [65]. Thus, neutrophil migration and function seem to be mediated by local levels of S1P in tissues. Unfortunately the source of increased tissue S1P during inflammation has not been explored, although it might be a good target for specific immunomodulation.

3.2. Monocytes, macrophages and dendritic cells

As for neutrophils, research on monocytes, macrophages and dendritic cells has primarily focused on the role of S1P receptors, while the role of S1P transporters is still unclear. Monocytes can migrate into tissues and differentiate on demand into macrophages and dendritic cells [53]. Immature dendritic cells (DCs) are present in nearly all kinds of tissue as highly efficient antigen presenting cells [66]. DCs continuously migrate from the periphery to lymph nodes, where they interact with T cells.

S1PR modulators (FTY720 and BAF312) under normal and inflammatory conditions restrict egress of monocytes from hematopoietic organs (spleen, bone marrow) and thereby reduce the number of circulating monocytes [60,67]. These findings resemble the effects of FTY720 on lymphocytes (see also section 2). In contrast to lymphocytes and monocytes, DCs are retained in blood and depleted in hematopoietic organs upon administration of FTY720 in mice. Moreover, FTY720 decreases expression of DC-adhesion molecules and impairs chemotactic responses [68]. The impaired migration of immature DCs from blood towards the spleen could result in spleen DC depletion since these cells have a short half-life. On the other hand, the S1PR₁ agonist SEW2871 increases the number of DC in the blood as well, but only for a limited time (< 24 hours) and without depleting DCs in hematopoietic organs. It is hypothesized that the difference in effects of FTY720 and SEW2871 is due to downregulation of S1PR₁ by FTY720-induced S1PR₁ internalization and degradation. Thus, stimulation of S1PR₁ seems to increase recirculation of DCs through an initial increase in the number of circulating DCs in blood.

In vitro S1P is chemotactic for immature, but not mature human DCs [69]. In contrast to human DCs, murine DCs upregulate the expression of S1PR₁ and S1PR₃ upon maturation. This leads to increased sensitivity for and migration towards S1P in mature DCs [70]. Although it cannot be excluded that the differential effect of S1P on mature DCs is due to technical differences, this is likely due to differences in species or in the source of the cells (blood versus bone marrow).

3.3. Mast cells

Mast cells are early sentinels of immune activation that have a role in fine-tuning of the immune response through release of pro- as well as anti-inflammatory substances [71]. A range of mast cell functions are regulated by S1P signaling, as reviewed in detail by Olivera et al. [56]. Stimulation of mast cells by allergens and IgE results in SphK1 activation and subsequent S1P production [72,73]. SphK1 is involved in anaphylaxis through the production of intracellular S1P by mast cells as well as production of circulating S1P from other sources, such as endothelial cells and platelets [73]. Intracellularly produced S1P can be exported from mast cells by ABCC1 (see also section 1.1) [12]. However, mast cells are not major contributors to the maintenance of a S1P gradient in the blood, since mast cell deficient mice (W^{-sh}/W^{-sh}) do not have altered S1P levels in the blood, while reconstitution of mast cells in these mice has no effect on the blood S1P concentration either [73].

Inhibition of the SphK1 completely prevents migration of mast cells towards antigen, while preserving their ability to migrate to S1P [72]. This suggests the migration of mast cells towards antigen depends on the production of S1P via SphK1 (Figure 2B). Interestingly, knockout of *Sphk1* in

mice leads to lower circulating S1P and resistance to anaphylaxis, a process in which mast cells have an important role [73]. However, *Sphk2*^{-/-} mice did develop anaphylaxis. The different anaphylactic responses in these two mice strains may depend on the plasma level of S1P, possibly being low in *Sphk1*^{-/-} mice, but high in *Sphk2*^{-/-} mice (see also section 3.1) [63,73]. It has been suggested that the increased plasma S1P level in *Sphk2*^{-/-} mice results in quick recovery from anaphylaxis and fast clearance of the histamine (one of the inflammatory mediators released by mast cells) [73]. High S1P promotes vasculature integrity and increases blood pressure through S1P receptors and thereby increases histamine clearance and attenuates anaphylaxis. The differential effects of *Sphk1*^{-/-} and *Sphk2*^{-/-} on mast cell function and anaphylaxis illustrates the redundancy in S1P-signaling, with contrary responses to locally versus systemically produced S1P, which hampers the discovery of novel pharmaceutical targets for resolution of inflammatory responses such as anaphylaxis. Thus, mast cell activity seems to depend on sphingosine kinases, although it remains to be unravelled whether this is mediated by the plasma S1P level, intracellular production of S1P in mast cells or both.

Extracellular S1P transactivates S1PR₁ and/or S1PR₂ [72]. The S1PR₂, but not the S1PR₁ is upregulated after FcεRI cross-linking. S1PR₁ activation leads to migration of mast cells towards antigen, while activation of S1PR₂ induces degranulation and inhibits mast cell motility (Figure 2B). These supplementary effects optimize mast cell function: S1PR₁ activation leads to mast cell migration towards antigen, whereas at the inflammatory site upregulation of S1PR₂ by antigen leads to cessation of migration and site-specific degranulation [72,74].

In conclusion, S1P seems to be involved in a wide range of leukocyte functions, including migration towards an inflammatory site. However, it is still not precisely clear whether the plasma level of S1P or local levels of S1P (i.e. in tissue or secondary lymphoid organs) are the primary determinants for functional modulation of different subtypes of leukocytes. Further research may enlighten the complex interactions between different immune cells in S1P-dependent signaling and elicit new targets for immunomodulation.

4. Conclusion

So far members of two families of proteins are known to function as S1P transporters, i.e. ABC transporters and SPNS2. A recurrent theme in literature is that disruption of a certain S1P transporter leads to incomplete inhibition of S1P release, indicating redundancy through compensation by other transporters. Moreover, the exact nature of the S1P transporter in erythrocytes and platelets remains elusive, although it shows characteristics of ABC transporters. In view of these notions, it is likely that additional S1P transporters will be identified in future research. These could include other ABC transporter family members, other members of the MSF (although SPNS1 appears to be excluded [35]), but it would not be surprising if entirely novel S1P transporters will be identified. The S1P transporters have an important role in establishing S1P gradients in the body that are important for immune system function. This includes the regulation of leukocyte migration and function in the conditions of homeostasis as well as inflammation. However, it remains to be clarified whether the absolute level of S1P in plasma or the local production of S1P resulting in a S1P gradient between different compartments is the primary determinant and how this affects different subtypes of leukocytes.

Conflict of Interest

All authors declare no conflicts of interest in this paper.

Supplementary

Table S1. Location and function of S1P transporters.

Name (Synonym)	Cell Type	Function	Reference
ABCA1	astrocytes	HDL loading	[17]
(ABC1, TBD, HDLDT1, CERP)	human vascular endothelial cells	plasma-tissue S1P gradient	[18]
ABCA7	platelets	lymphocyte egress	[27]
	erythrocytes	lymphocyte egress	[28]
ABCC1	mast cells	migration	[12]
(MRP1, MRP, GSX)	leiomyoma ELT3 and late pregnant rat myometrium	cell growth	[13]
	fibroblasts	cytoprotection	[14]
	brain and spinal cord endothelial cells	blood-brain barrier	[15]
	MCF7 human breast cancer cells	cancer/multidrug resistance	[16]
ABCG2	MCF7 human breast cancer cells	cancer/multidrug resistance	[16]
zebrafish SPNS2	extraembryonic tissue yolk syncytial layer	migration myocardial precursors	[34,35]
(Spinster 2)			
murine SPNS2	endothelial cells	lymphocyte egress	[37-41]
(Spinster 2)	osteoblasts	paracrine proliferation/survival	[42]

Table summarizes the known transporters for S1P, where they are localized and in which function they play a role, with references to the literature.

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